MycoKeys 68: 1–21 (2020)
doi: 10.3897/mycokeys.68.51667
http://mycokeys.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
MycoKeys
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Three new *Curvularia* species from clinical and environmental sources

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Academic editor: N. Wijayawardene | Received 3 March 2020 | Accepted 18 April 2020 | Published 17 June 2020

Citation: Iturrieta-González I, Gené J, Wiederhold N, García D (2020) Three new *Curvularia* species from clinical and environmental sources. MycoKeys 68: 1–21. https://doi.org/10.3897/mycokeys.68.51667

Abstract

Curvularia is a Pleosporalean monophyletic genus with a great diversity of species, including relevant phytopathogenic, animal and human pathogenic fungi. However, their microscopic identification is difficult due to overlapping morphological features amongst species. In recent years, multi-locus sequence analysis using the ITS region of the rDNA and fragments of the genes *gapdh* and *tef*1 revealed numerous cryptic species, especially in isolates that commonly produced 3-septate conidia. Therefore, based on sequence analysis of the above-mentioned DNA barcodes recommended for species delineation in *Curvularia*, we propose three novel species, *C. paraverruculosa*, *C. suttoniae* and *C. vietnamensis*, isolated from soil, human clinical specimens and plant material, respectively, collected in different countries. These new species are morphologically characterised and illustrated in the present study. *Curvularia paraverruculosa* differs from its counterparts, *C. americana* and *C. verruculosa*, mainly by its narrower conidia. *Curvularia suttoniae* and *C. vietnamensis* are closely related to *C. petersonii*, but the former two have larger conidia.

Keywords

Ascomycetes, Dematiaceous hyphomycetes, phylogeny, Pleosporaceae, taxonomy

Introduction

The genus *Curvularia* Boedijn (1933), typified by *C. lunata* (Wakker) Boedijn, belongs in Pleosporaceae, Pleosporales (Wijayawardene et al. 2018). Members of *Curvularia* show different life modes, i.e. saprophytic, endophytic and also pathogenic on plants and animals (Marin-Felix et al. 2017a). Phytopathogenic species can affect wild grasses

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and staple crops, such as rice, maize, wheat or sorghum and give rise to serious losses in agricultural production (Gautam et al. 2013, Manamgoda et al. 2015, Marin-Felix et al. 2017a, Tan et al. 2018). The endophytic species have garnered interest in recent years for their use in the production of bio-based products that are beneficial to living organisms and the environment (Bengyella et al. 2019). Since the first report of *Curvularia* as a human pathogen in a patient with mycetoma (Baylet et al. 1959), other clinical presentations have been reported, such as superficial and deep infections that mainly affect the respiratory tract but can even cause cerebral phaeohyphomycosis with an extremely poor prognosis (de Hoog et al. 2000).

The genus is morphologically distinguished mainly by its asexual morph, which shows sympodial conidiophores with mono- to polytretic conidiogenous cells and transversally septate conidia. Typically, the conidia in *Curvularia* are curved due to the hypertrophy of one of the intermediate cells and they are euseptate (Ellis 1971), although other authors opine that the conidia in *Curvularia* are distoseptate (Sivanesan 1987, Seifert et al. 2011, Madrid et al. 2014). The species of *Bipolaris* and *Exserohilum* have typically straight and distoseptate conidia; however, some of them have been transferred to *Curvularia*, based on their DNA sequence analyses (Manamgoda et al. 2012, Hernández-Restrepo et al. 2018, Tan et al. 2018). Furthermore, due to the overlapping of morphological characters amongst certain species of *Curvularia*, such as conidial size, shape and septation, an accurate identification at the species level is difficult without a DNA sequence analysis (da Cunha et al. 2013, Madrid et al. 2014, Manamgoda et al. 2015). Several cryptic species have been described recently using only multi-locus sequence analyses of the recommended DNA barcodes for species delimitation, i.e. the internal transcribed spacer (ITS) region of the rDNA and the protein-coding loci glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and translation elongation factor 1-a (*tef*1) (Marin-Felix et al. 2017a, Tan et al. 2018). Nearly 130 species have so far been accepted in *Curvularia*, including the species classified previously in the teleomorphic genera *Cochliobolus* and *Pseudocochliobolus* after applying the current criteria for fungal nomenclature (Manamgoda et al. 2012, 2015, Madrid et al. 2014, Hyde et al. 2017, Marin-Felix et al. 2017a, 2017b, Dehdari et al. 2018, Heidari et al. 2018, Hernández-Restrepo et al. 2018, Liang et al. 2018, Mehrabi-Koushki et al. 2018, Tan et al. 2018, Tibpromma et al. 2018, Kiss et al. 2019, Raza et al. 2019, Zhang et al. 2020).

Based on a polyphasic approach, combining morphological and phylogenetic analyses, three novel *Curvularia* species are proposed here, isolated from human clinical specimens in the USA, soil in Mexico and seed and plant debris in Vietnam and Indonesia, respectively.

Material and methods

Origin of isolates

Five unidentified *Curvularia* isolates, maintained in the fungal collection of the Medical School of the Rovira i Virgili University (FMR; Reus, Spain), were included in the

study. Two of these (FMR 10992, FMR 11690) were isolated from human specimens in the USA by Deana A. Sutton of the Fungus Testing Laboratory at the University of Texas Health Sciences Center (UTHSC; San Antonio, USA) and the other three (FMR 11956, FMR 17656, FMR 17659) were isolated from environmental samples; the first from sorghum seeds collected in Indonesia, the second from soil collected in the Mexican region of Michoacán and the third from unidentified plant material collected in the north-east of Vietnam.

DNA extraction, PCR, sequencing and phylogenetic analysis

The fungal DNA was extracted from colonies growing on potato dextrose agar (PDA; Pronadisa, Madrid, Spain) for 7 to 10 days at 25 °C in darkness and following the protocol of Müller et al. (1998). The ITS barcode, including the 5.8S gene and the genes *gapdh* and *tef*1 were analysed following Marin-Felix et al. (2017a). Amplification was carried out using the primer pairs ITS5/ITS4 for the ITS region (White et al. 1990), gpd1/gpd2 for *gapdh* (Berbee et al. 1999) and EF983/2218R for *tef*1 (Schoch et al. 2009). The PCR products were purified and stored at -20 °C until sequencing. The same pairs of primers used for the amplification were also used to obtain the DNA sequences, which were processed at Macrogen Europe (Macrogen Inc., Madrid, Spain). The sequences of each isolate were edited using SeqMan v. 7.0.0 (DNAStar Lasergene, Madison, WI, USA) to obtain the consensus sequences.

We made a preliminary comparison of *gapdh* sequences generated from our isolates with those of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLASTn) for their molecular identification. To establish the phylogenetic position of unidentified isolates with respect to the most accepted species in *Curvularia*, we carried out individual (data not shown) and combined alignments of the three loci complemented by all available sequences of the ex-type and reference strains of *Curvularia* species retrieved from NCBI (Table 1). Based on this first phylogeny of the genus, a more restricted multi-locus analysis was carried out, including only those *Curvularia* species most related to the isolates under study. The alignments were made in the MEGA (Molecular Evolutionary Genetics Analysis) software v.6.0. (Tamura et al. 2013), using ClustalW algorithm (Thompson et al. 1994), refined with MUSCLE (Edgar 2004) in the same platform and manually adjusted as necessary. Phylogenetic reconstructions were made using Maximum Likelihood (ML) and Bayesian Inference (BI) approaches under RAxML-HPC2 on XSEDE v.8.2.12 (Stamatakis et al. 2014) in CIPRES Science gateway portal (Miller et al. 2010) and MrBayes v. 3.2.6 (Ronquist et al. 2012), respectively.

For the ML analysis, the best nucleotide substitution model for the combined analysis of ITS, *gapdh* and *tef*1, determined using the MEGA programme, was Kimura 2-parameters with Gamma distribution (K2+G); the combined analysis of these three phylogenetic markers was tested through Incongruence Length Difference (ILD) implemented in the Winclada programme (Farris et al. 1994). ML bootstrap values (bs) ≥ 70% were considered significant.

Table 1. Species included in this study, their substrate, origin and GenBank accession numbers.

Species	Strain no[1]	Substrate	Country	Genbank accession no.[2]		
				ITS	*gapdh*	*tef*1
Bipolaris maydis	CBS 136.29 T	*Zea mays*	USA	AF071325	KM034846	KM093794
B. saccharicola	CBS 155.26 T	Unknown	Unknown	KY905674	KY905686	KY905694
Curvularia aeria	CBS 294.61 T	Air	Brazil	HF934910	HG779148	–
C. affinis	CBS 154.34 T	Unknown	Indonesia	KJ909780	KM230401	KM196566
C. ahvazensis	CBS 144673 T	*Zinnia elegans*	Iran	KX139029	MG428693	MG428686
C. akaii	CBS 317.86	*Themada triandra* subsp. *japonica*	Japan	KJ909782	KM230402	KM196569
C. akaiiensis	BRIP 16080 T	Unknown	India	KJ415539	KJ415407	KJ415453
C. alcornii	MFLUCC 10-0703 T	*Z. mays*	Thailand	JX256420	JX276433	JX266589
C. americana	UTHSC 08-3414 T	Human ankle	USA	HE861833	HF565488	–
	UTHSC 07-2649	Human toe tissue	USA	HE861834	HF565486	–
	UTHSC 08-84	Human nasal sinus	USA	HG779015	HG779115	–
	UTHSC 08-278	Human peritoneal dialysis fluid	USA	HE861832	HF565487	–
	UTHSC 08-2697	Human leg	USA	HG779016	HG779117	–
C. annelliconidiophori	CGMCC3.19352 T	Roots of *Saccharum officinarum*	China	MN215641	MN264077	MN263935
C. asiatica	MFLUCC 10-0711 T	*Panicum* sp.	Thailand	JX256424	JX276436	JX266593
C. australiensis	BRIP 12044 T	*Oryza sativa*	Australia	KJ415540	KJ415406	KJ415452
	CBS 172.57	*O. sativa* seeds	Vietnam	JN601026	JN601036	JN601003
C. australis	BRIP 12521 T	*Sporobolus caroli*	Australia	KJ415541	KJ415405	KJ415451
C. bannonii	BRIP 16732 T	*Jacquemontia tamnifolia*	USA	KJ415542	KJ415404	KJ415450
C. beasleyi	BRIP 10972 T	*Chloris gayana*	Australia	MH414892	MH433638	MH433654
	BRIP 15854	*Leersia hexandra*	Australia	MH414893	MH433639	MH433655
C. beerburrumensis	BRIP 12942 T	*Eragrostis bahiensis*	Australia	MH414895	MH433634	MH433657
C. boeremae	IMI 164633 T	*Portulaca oleracea*	India	MH414911	MH433641	–
C. borreriae	CBS 859.73	Volcanic ash soil	Chile	HE861848	HF565455	–
C. bothriochloae	BRIP 12522 T	*Bothriochloa bladhii*	Australia	KJ415543	KJ415403	KJ415449
C. brachyspora	CBS 186.50	Soil	Indonesia	KJ922372	KM061784	KM230405
C. buchloes	CBS 246.49 T	*Buchloë dactyloides*	USA	KJ909765	KM061789	KM196588
C. carica-papayae	CBS 135941 T	*Carica papaya*	India	HG778984	HG779146	–
C. chiangmaiensis	CPC 28829 T	*Z. mays*	Thailand	MF490814	MF490836	MF490857
C. chlamydospora	UTHSC 07-2764 T	Human toe nail	USA	HG779021	HG779151	–
C. chonburiensis	MFLUCC 16-0375 T	Dead leaf of *Pandanus* sp.	Thailand	MH275055	MH412747	–
C. clavata	BRIP 61680	*Oryza* sp.	Australia	KU552205	KU552167	KU552159
C. cymbopogonis	CBS 419.78	*Yucca* leaf spot	Netherlands	HG778985	HG779129	–
C. coatesiae	BRIP 24261 T	*Litchi chinensis*	Australia	MH414897	MH433636	MH433659
C. coicis	CBS 192.29 T	*Coix lacryma-jobi*	Japan	AF081447	AF081410	JN601006
C. coimbatorensis	SZMC 22225 T	Human corneal scraping	India	MN628310	MN628306	MN628302
C. colbranii	BRIP 13066 T	*Crinum zeylanicum*	Australia	MH414898	MH433642	MH433660
C. comoriensis	CBS 110673	Unknown	Unknown	LT631357	LT715841	–

Species	Strain no[1]	Substrate	Country	Genbank accession no.[2]		
				ITS	*gapdh*	*tef*1
C. crassiseptum	CBS 503.90 T	Plant material	Nigeria	LT631310	LT715882	–
C. crustacea	BRIP 13524 T	*Sporobolus* sp.	Indonesia	KJ415544	KJ415402	KJ415448
C. dactyloctenicola	CPC 28810 T	*Dactyloctenium aegyptium*	Thailand	MF490815	MF490837	MF490858
C. dactyloctenii	BRIP 12846 T	*Dactyloctenium radulans*	Australia	KJ415545	KJ415401	KJ415447
C. deightonii	CBS 537.70	*Sorghum vulgare*	Denmark	LT631356	LT715839	–
C. determinata	CGMCC3.19340 T	Leaves of *S. officinarum*	China	MN215653	MN264088	MN263947
C. elliptiformis	CGMCC3.19351 T	Roots of *S. officinarum*	China	MN215656	MN264091	MN263950
C. ellisii	CBS 193.62 T	Air	Pakistan	JN192375	JN600963	JN601007
C. eragrosticola	BRIP 12538 T	*Eragrostis pilosa*	Australia	MH414899	MH433643	MH433661
C. eragrostidis	CBS 189.48	*Sorghum* seed	Indonesia	HG778986	HG779154	–
C. falsilunata	CGMCC3.19329 T	Roots of *S. officinarum*	China	MN215660	MN264093	MN263954
C. flexuosa	CGMCC3.19447 T	Roots of *S. officinarum*	China	MN215663	MN264096	MN263957
C. gladioli	CBS 210.79	Gladiolus leaf	Romania	HG778987	HG779123	–
C. geniculata	CBS 187.50	*Andropogon sorghum* seed	Indonesia	KJ909781	KM083609	KM230410
C. graminícola	BRIP 23186 T	*Aristida ingrata*	Australia	JN192376	JN600964	JN601008
C. guangxiensis	CGMCC3.19330 T	Roots of *S. officinarum*	China	MN215667	MN264100	MN263961
C. gudauskasii	DAOM 165085	Unknown	Unknown	AF071338	AF081393	–
C. harveyi	BRIP 57412 T	*Triticum aestivum*	Australia	KJ415546	KJ415400	KJ415446
C. hawaiiensis	BRIP 11987 T	*O. sativa*	USA	KJ415547	KJ415399	KJ415445
C. heteropogonicola	BRIP 14579 T	*Heteropogon contortus*	India	KJ415548	KJ415398	KJ415444
C. heteropogonis	CBS 284.91 T	*H. contortus*	Australia	KJ415549	JN600969	JN601013
C. hominis	CBS 136985 T	Human cornea	USA	HG779011	HG779106	–
C. homomorpha	CBS 156.60 T	Air	USA	JN192380	JN600970	JN601014
C. inaequalis	CBS 102.42 T	Soil	France	KJ922375	KM061787	KM196574
C. intermedia	CBS 334.64	*Avena versicolor*	USA	HG778991	HG779155	–
C. ischaemi	CBS 630.82 T	*Ischaemum indicum*	Solomon Islands	MH861533	JX276440	–
C. kenpeggii	BRIP 14530 T	*Triticum aestivum*	Australia	MH414900	MH433644	MH433662
C. kusanoi	CBS 137.29	*Eragrostis major*	Japan	JN192381	LT715862	JN601016
C. lamingtonensis	BRIP 12259 T	*Microlaena stipoides*	Australia	MH414901	MH433645	MH433663
C. lunata	CBS 730.96 T	Human lung biopsy	USA	JX256429	JX276441	JX266596
C. malina	CBS 131274 T	*Zoysia matrella*	USA	JF812154	KP153179	KR493095
C. manamgodae	CGMCC3.19446 T	Roots of *S. officinarum*	China	MN215677	MN264110	MN263971
	LC13495	Roots of *S. officinarum*	China	MN215678	MN264111	MN263972
C. mebaldsii	BRIP 12900 T	*Cynodon transvaalensis*	Australia	MH414902	MH433646	MH433664
	BRIP 13983	*Cynodon dactylon* x *C. transvaalensis*	Australia	MH414903	MH433647	MH433665
C. micropus	CBS 127235 ET	*Paspalum notatum*	Georgia	HE792934	LT715859	–

Species	Strain no[1]	Substrate	Country	Genbank accession no.[2]		
				ITS	*gapdh*	*tef*1
C. microspora	GUCC 6272 T	*Hippeastrum striatum*	China	MF139088	MF139106	MF139115
C. miyakei	CBS 197.29 T	*Eragrostis pilosa*	Japan	KJ909770	KM083611	KM196568
C. mosaddeghii	IRAN 3131C T	*Syzygium cumini*	Iran	MG846737	MH392155	MH392152
C. muehlenbeckiae	CBS 144.63 T	*Sorghum* sp.	USA	MH858242	HG779108	KM196578
C. neergaardii	BRIP 12919 T	*O. sativa*	Ghana	KJ415550	KJ415397	KJ415443
	CBS 276.91	Unknown	Australia	LT631362	LT715848	–
C. neoindica	IMI 129790 T	*Brassica nigra*	India	MH414910	MH433649	MH433667
C. nicotiae	BRIP 11983 T	Soil	Algeria	KJ415551	KJ415396	KJ415442
C. nodosa	CPC 28800 T	*Digitaria ciliaris*	Thailand	MF490816	MF490838	MF490859
	CPC 28801	*Brachiaria reptans*	Thailand	MF490817	MF490839	MF490860
C. nodulosa	CBS 160.58	*Eleusine indica*	Unknown	JN601033	JN600975	JN601019
C. oryzae	CBS 169.53 T	*O. sativa*	Vietnam	KP400650	KP645344	KM196590
C. ovariicola	CBS 470.90 T	*Eragrostis interrupta*	Australia	JN192384	JN600976	JN601020
C. pallescens	CBS 156.35 T	Air	Indonesia	KJ922380	KM083606	KM196570
C. palmicola	MFLUCC 14-0404 T	Dead branches of *Acoelorrhaphe wrightii*	Thailand	MF621582	–	–
C. pandanicola	MFLUCC 15-0746 T	Dead leaf of *Pandanus* sp.	Thailand	MH275056	MH412748	MH412763
C. papendorfii	CBS 308.67 T	*Acacia karroo*	South Africa	KJ909774	KM083617	KM196594
C. paraverruculosa	**FMR 17656 T**	**Soil**	**Mexico**	**LR736641**	**LR736646**	**LR736649**
C. petersonii	BRIP 14642 T	*D. aegyptium*	Australia	MH414905	MH433650	MH433668
C. perotidis	CBS 350.90 T	*Perotis rara*	Australia	JN192385	KJ415394	KM230407
C. phaeospara	CGMCC3.19448 T	Roots of *S. officinarum*	China	MN215686	MN264118	MN263980
C. pisi	CBS 190.48 T	*Pisum sativum*	Canada	KY905678	KY905690	KY905697
C. plantarum	CGMCC3.19342 T	Roots of *S. officinarum*	China	MN215688	MN264120	MN263982
C. platzii	BRIP 27703b T	*Cenchrus clandestinum*	Australia	MH414906	MH433651	MH433669
C. polytrata	CGMCC3.19338 T	Roots of *S. officinarum*	China	MN215691	MN264123	MN263984
C. portulacae	BRIP 14541 T	*Portulaca oleracea*	USA	KJ415553	KJ415393	KJ415440
C. prasadii	CBS 143.64 T	*Jasminum sambac*	India	KJ922373	KM061785	KM230408
C. protuberans	CGMCC3.19360 T	Leaves of *S. officinarum*	China	MN215693	MN264125	MN263986
C. protuberata	CBS 376.65 T	*Deschampsia flexuosa*	UK	KJ922376	KM083605	KM196576
C. pseudobrachyspora	CPC 28808 T	*Eleusine indica*	Thailand	MF490819	MF490841	MF490862
C. pseudolunata	UTHSC 09-2092 T	Human nasal sinus	USA	HE861842	HF565459	–
C. pseudorobusta	UTHSC 08-3458	Human nasal sinus	USA	HE861838	HF565476	–
C. radici-foliigena	CGMCC3.19328 T	Roots of *S. officinarum*	China	MN215695	MN264127	MN263988
	LC11956	Roots of *S. officinarum*	China	MN215698	MN264130	MN263991
C. radicicola	CGMCC3.19327 T	Roots of *S. officinarum*	China	MN215699	MN264131	MN263992
	LC11953	Roots of *S. officinarum*	China	MN215700	MN264132	MN263993
C. ravenelii	BRIP 13165 T	*Sporobolus fertilis*	Australia	JN192386	JN600978	JN601024
C. reesii	BRIP 4358 T	Air	Australia	MH414907	MH433637	MH433670

Species	Strain no[1]	Substrate	Country	Genbank accession no.[2]		
				ITS	*gapdh*	*tef*1
C. richardiae	BRIP 4371 T	*Richardia brasiliensis*	Australia	KJ415555	KJ415391	KJ415438
C. robusta	CBS 624.68 T	*Dichanthium annulatum*	USA	KJ909783	KM083613	KM196577
C. rouhanii	CBS 144674 T	*Syngonium vellozianum*	Iran	KX139030	MG428694	MG428687
C. ryleyi	BRIP 12554 T	*Sporobolus creber*	Australia	KJ415556	KJ415390	KJ415437
C. saccharicola	CGMCC3.19344 T	Roots of *S. officinarum*	China	MN215701	MN264133	MN263994
C. sacchari-officinarum	CGMCC3.19331 T	Leaves of *S. officinarum*	China	MN215705	MN264137	MN263998
C. senegalensis	CBS 149.71	Unknown	Nigeria	HG779001	HG779128	–
C. shahidchamranensis	IRAN 3133C T	Crude oil contaminated soil	Iran	MH550084	MH550083	–
C. soli	CBS 222.96 T	Soil	Papua New Guinea	KY905679	KY905691	KY905698
C. sorghina	BRIP 15900 T	*Sorghum bicolor*	Australia	KJ415558	KJ415388	KJ415435
C. spicifera	CBS 198.31	*Capsicum anuum*	Cyprus	HF934916	HG779136	–
	CBS 274.52	Soil	Spain	JN192387	JN600979.	JN601023
C. sporobolicola	BRIP 23040b T	*Sporobolus australasicus*	Australia	MH414908	MH433652	MH433671
C. subpapendorfii	CBS 656.74 T	Soil	Egypt	KJ909777	KM061791	KM196585
C. suttoniae	**FMR 10992 T**	**Human leg wound**	**USA**	HE861828	HF565479	**LR736651**
	FMR 11690	**Human sphenoid sinus**	**USA**	HE861826	HF565477	**LR736650**
C. tamilnaduensis	SZMC 22226 T	Human corneal scraping	India	MN628311	MN628307	MN628303
	SZMC 26758	Human corneal scraping	India	MN628308	MN628304	MN628300
	SZMC 26759	Human corneal scraping	India	MN628309	MN628305	MN628301
C. thailandicum	MFLUCC 15-0747 T	Decaying leaves of *Pandanus* sp.	Thailand	MH275057	MH412749	MH412764
C. trifolii	CBS 173.55	*Trifolium repens*	USA	HG779023	HG779124	–
C. tripogonis	BRIP 12375 T	*Tripogon loliiformis*	Australia	JN192388	JN600980	JN601025
C. tropicalis	BRIP 14834 T	*Coffea arabica*	India	KJ415559	KJ415387	KJ415434
C. tsudae	ATCC 44764 T	*Chloris gayana*	Japan	KC424596	KC747745	KC503940
	BRIP 10967	Leaf tip blight of *C. gayana*	Australia	KC424604	KC747754	KC503949
C. tuberculata	CBS 146.63 T	*Z. mays*	India	JX256433	JX276445	JX266599
C. umbiliciformis	CGMCC3.19346 T	Roots of *S. officinarum*	China	MN215711	MN264142	MN264004
C. uncinata	CBS 221.52 T	*O. sativa*	Vietnam	HG779024	HG779134	–
C. variabilis	CPC 28815 T	*Chloris barbata*	Thailand	MF490822	MF490844	MF490865
	CPC 28816	*Imperata cylindrica*	Thailand	MF490823	MF490845	MF490866
C. verruciformis	CBS 537.75	*Lobibyx* sp. feather	New Zealand	HG779026	HG779133	–
C. verruculosa	CBS 149.63	*Elaeis guineensis*	Nigeria	HF934909	HG779110	–
	CBS 150.63	*Punica granatum* leaf	India	KP400652	KP645346	KP735695
	CPC 28792	*C. dactylon*	Thailand	MF490825	MF490847	MF490868
	CPC 28809	*E. indica*	Thailand	MF490824	MF490846	MF490867

Species	Strain no[1]	Substrate	Country	Genbank accession no.[2]		
				ITS	*gapdh*	*tef*1
C. vietnamensis	**FMR 17659 T**	**Unidentified dead leaves**	**Vietnam**	**LR736642**	**LR736644**	**LR736647**
	FMR 11956	***Sorghum* seed**	**Indonesia**	**LR736652**	**LR736643**	**LR736648**
C. warraberensis	BRIP 14817 T	*D. aegyptium*	Australia	MH414909	MH433653	MH433672
C. xishuangbannaensis	KUMCC 17-0185 T	Decaying leaves of *Pandanus amaryllifollus*	China	MH275058	MH412750	MH412765

[1] ATCC: American Type Culture Collection, Virginia, USA; BRIP: Queensland Plant Pathology Herbarium, Brisbane, Australia; CBS: Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CGMCC: China General Microbiological Culture Collection Center, China; CPC: Culture collection of Pedro Crous, housed at Westerdijk Fungal Biodiversity Institute; DAOM: Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; FMR: Facultat de Medicina, Universitat Rovira i Virgili, Reus, Spain; GUCC: Department of Plant Pathology, Agriculture College, Guizhou University, P.R. China; IMI: International Mycological Institute, Kew, UK; IRAN: Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Iran; KUMCC: Culture Collection of Kunming Institute of Botany, Kunming, China; LC: Personal culture collection held in the laboratory of Prof. Lei Cai, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Ria, Thailand; MUCL: Mycothe`que de l'Universite´ Catholique de Louvain, Louvain-la-Neuve, Belgium; SZMC: Szeged Microbiological Collection at the Department of Microbiology, Faculty if Science and Informatics, University of Szeged, Hungary; UTHSC: Fungus Testing Laboratory, Department of Pathology at the University of Texas Health Science Center, San Antonio, Texas, USA. T and ET indicate ex-type and ex-epitype strain.

[2]Sequences newly generated in this study and novel species proposed are indicated in bold.

For the BI phylogenetic analysis, the best nucleotide substitution model was determined using jModelTest (Posada 2008). For the ITS region, we used Kimura 2-parameter with Invariant sites (K80+I), for *gapdh* General Time Reversible with gamma distribution (GTR+G) and for *tef*1 General Time Reversible with invariant sites (GTR+I). The parameter settings used were two simultaneous runs of 5M generations, four Markov chains, sampled every 1000 generations. The 50% majority-rule consensus tree and posterior probability values were calculated after discarding the first 25% of the samples. A posterior probability (pp) value of ≥ 0.95 was considered significant.

Sequence data generated in the present study were deposited in GenBank (Table 1) and the alignments in TreeBASE (http://treebase.org).

Phenotypic study

Macroscopic characterisation of the colonies was made on PDA, oatmeal agar (OA; oatmeal 30 g, agar 13 g, distilled water 1 litre) and potato carrot agar (PCA; potato 20 g, carrot 20 g, agar 13 g, distilled water 1 litre), after 7 days at 25 °C in darkness. Colours of the colonies in descriptions were based on Kornerup & Wanscher (1978). Cardinal temperatures for growth were obtained on PDA after 7 days in darkness.

Microscopic features were studied from the specimens mounted in Shear's solution growing on the same media (Madrid et al. 2014). At least 30 measurements were taken for the calculation of conidial and conidiophores length and width ranges, which are also reported as the mean plus or minus standard deviation in the descriptions.

Photomicrographs were taken using a Zeiss Axio-Imager M1 light microscope (Zeiss, Oberkochen, Germany) with a DeltaPix Infinity X digital camera.

Nomenclatural novelties and descriptions were deposited in MycoBank (Crous et al. 2004). Ex-type cultures and holotypes, which were dried cultures, were deposited at the Westerdijk Fungal Biodiversity Institute from Utrecht (CBS, The Netherlands).

Results

BLASTn results with *gapdh* sequences showed that the isolate FMR 17656 was ≤ 97.6%, similar to *C. verruculosa* CPC 28792; FMR 11956 and FMR 17659 showed a similarity of 93.31% and 93.6%, respectively, with *C. spicifera* CBS 198.31; and isolates FMR 10992 and FMR 11690 both exhibited a similarity of 94.7% with the ex-type strain of *C. petersonii* (BRIP 14642). Sequence similarity with this marker between FMR 11956/17659 and FMR 10992/11690 was 97%. These values suggested that the unidentified isolates represented putative new species for the genus, which were then confirmed by multi-locus sequence analysis of ITS, *gapdh* and *tef*1 barcodes. The combined analysis included 128 sequences representing 126 taxa in the genus *Curvularia* and these were rooted with *Bipolaris maydis* (CBS 136.29) and *B. saccharicola* (CBS 155.26) (Suppl. material 1: Fig. S1). The alignment comprised a total of 1928 bp (ITS 432, *gapdh* 573 bp and *tef*1 923 bp), including 546 variable sites (ITS 119 bp, *gapdh* 253 bp and *tef*1 174 bp) and 445 phylogenetically informative (ITS 83 bp, *gapdh* 233 bp and *tef*1 129 bp). The unidentified isolates were allocated to three single lineages in the same clade (74/0.99) together with sequences of the ex-type strains of *C. americana* (UTHSC 08-3414), *C. petersonii* (BRIP 14642) and *C. verruculosa* (CBS 150.63), but with enough distance to be considered distinct species. The two clinical isolates (FMR 10992 and FMR 11690) formed a fully-supported clade closely related to isolates FMR 11956 and FMR 17659, which were collected in Indonesia and Vietnam, respectively and to *C. petersonii*. The fifth isolate (FMR 17656) was related to *C. verruculosa* and *C. americana*, but formed an independent and distant branch from the previously-mentioned species.

In order to evaluate possible intra- and inter-specific variability within the species and to confirm the novelty of these fungi, we performed a multi-locus analysis, including only those sequences of the species that were more related to the unidentified *Curvularia* isolates (Fig. 1). The alignment comprised a total of 1894 bp (ITS 409, *gapdh* 562 bp and *tef*1 923 bp), with 298 variable sites (ITS 66 bp, *gapdh* 135 bp and *tef*1 97 bp) and 240 being phylogenetically informative (ITS 51 bp, *gapdh* 117 bp and *tef*1 72 bp). The phylogenetic analyses show that these isolates indeed represent three new species, which are described and illustrated in the Taxonomy section. The species can be morphologically differentiated mainly by features of their conidia (Table 2).

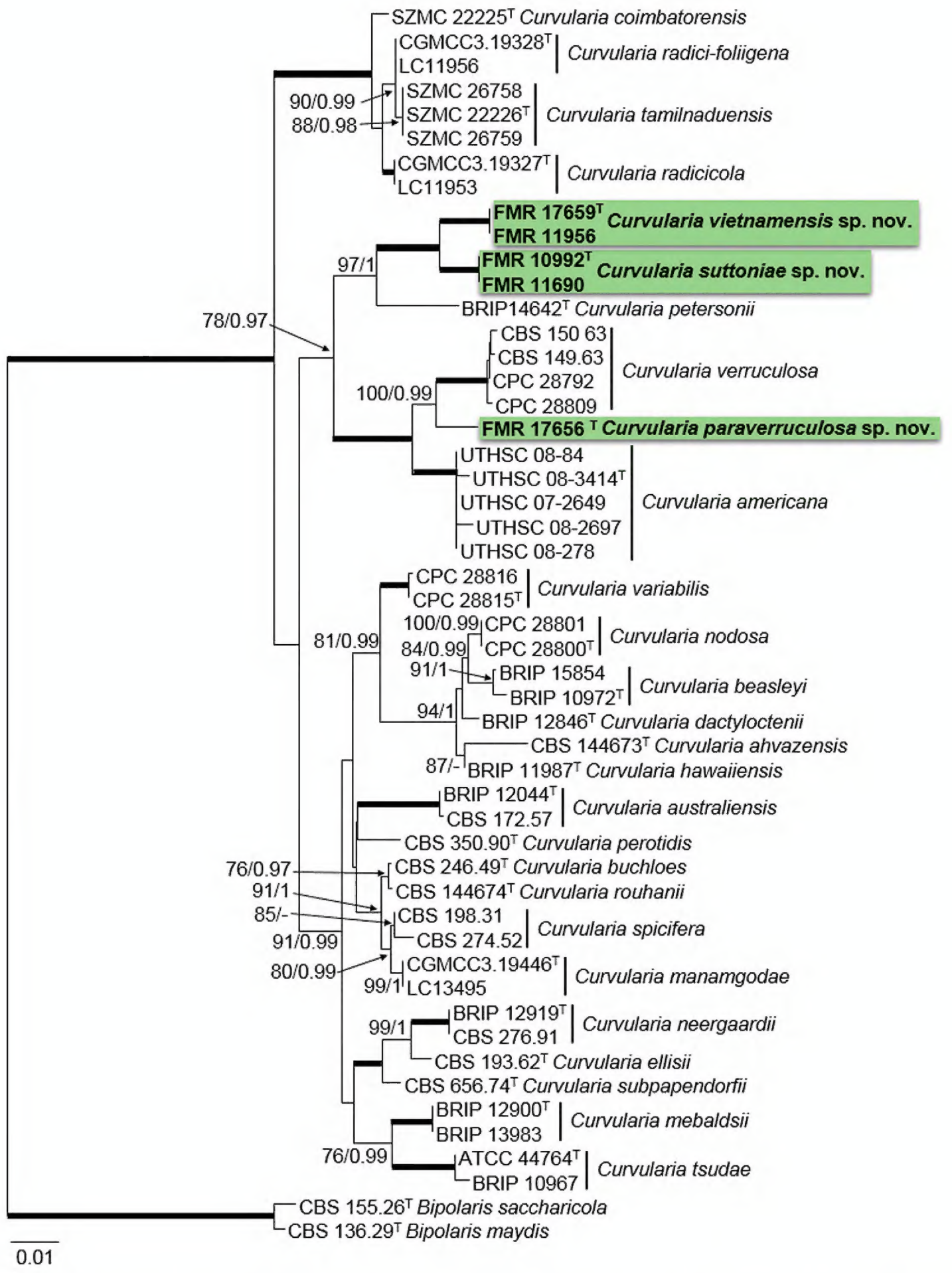

Figure 1. Phylogenetic tree of the *Curvularia* species most related to the new taxa based on Maximum Likelihood analysis obtained by RAxML, using the combined analysis of ITS, *gapdh* and *tef*1 and rooted with *Bipolaris maydis* CBS 136.29 and *Bipolaris saccharicola* CBS 155.26. Bootstrap values (bs) greater than 70% and Bayesian posterior probabilities (pp) greater than 0.95 are given at the nodes (bs/pp). Bold branches indicate bs/pp of 100/1. The novel species are highlighted in bold. Ex-type isolates are marked with a superscript T.

Table 2. Conidial features of the novel *Curvularia* species proposed here and of their closest relatives.

Species	Size (μm)	Septum no.	Ornamentation	References
C. americana	13–28 × 7–15	3–4	Smooth upper cells, verruculose basal cell	Madrid et al. (2014)
C. palmicola	23.9–34.7 × 9.3–15.7	3	Smooth	Hyde et al. (2017)
C. paraverruculosa	11–37 × 8–12	3(–4)	Verruculose to verrucose	Present study
C. petersonii	(15–)17–19(–21) × (5–)5.5–6(–7)	3	Smooth	Tan et al. (2018)
C. suttoniae	8–22 × 5–9	(2–)3	Smooth upper cells, verruculose basal cell	Present study
C. verruculosa	20–40 × 12–17	3	Rough to verruculose	Sivanesan (1987)
C. vietnamensis	15–28 × 5–12	(1–)3(–4)	Smooth	Present study

Taxonomy

Curvularia paraverruculosa **Iturrieta-González, Gené & Dania García, sp. nov.**
MycoBank No: 833024
Fig. 2

Etymology. Name refers to the phylogenetic closeness to *Curvularia verruculosa*.

Type. Mexico, Michoacán, Villa Jiménez, from soil, Sept 2016, *E. Rosas de Paz.* (holotype CBS H-24293, culture ex-type FMR 17656, CBS 146220).

Description (PDA at 25 °C). *Mycelium* composed of branched, septate, subhyaline to pale brown, thin- and smooth-walled hyphae, 2–4 μm wide. *Conidiophores* semi- to macronematous, mononematous, septate, straight or flexuous, geniculate at upper part, unbranched or slightly branched, smooth-walled, yellowish-brown to brown, 19–85(–145) × 3–6 μm (av. (±SD) 49.6 (±43.8) × 4.6 (±0.69)). *Conidiogenous cells* terminal or intercalary, polytretic, proliferating sympodially, yellowish-brown, with darkened scars, subcylindrical, 4–6 μm wide. *Conidia* 3(–4)-septate, mostly curved at the third cell from base which is usually larger than the others, sometimes apically bifurcate, verruculose to verrucose, apical and basal cells subhyaline to pale brown, middle cells brown, 11–37 × 8–12 μm (av. (±SD) 24 (±18.38) × 9.58 (±1.66)); hila slightly protuberant, thickened and darkened. Sexual morph not observed.

Culture characteristics (7 d at 25 °C). *Colonies* on PDA reaching 45 mm diam., dark green (30F8), final edge whitish, velvety, flat, margin regular and fimbriate; reverse dark green (30F8). On PCA and OA, reaching 58–60 mm diam., dark green (30F8), final edge whitish, slightly floccose, flat, margin regular and fimbriate; reverse dark green (30F8). Sporulation was abundant on the three media.

Cardinal temperature for growth. Optimum 30 °C, maximum 37 °C, minimum 15 °C.

Distribution. Mexico.

Notes. *Curvularia paraverruculosa* is allocated phylogenetically to a strongly-supported clade (100/1) with *C. verruculosa* and *C. americana* (Fig. 1). All three species commonly have 3-septate conidia, but these can be distinguished by their size and

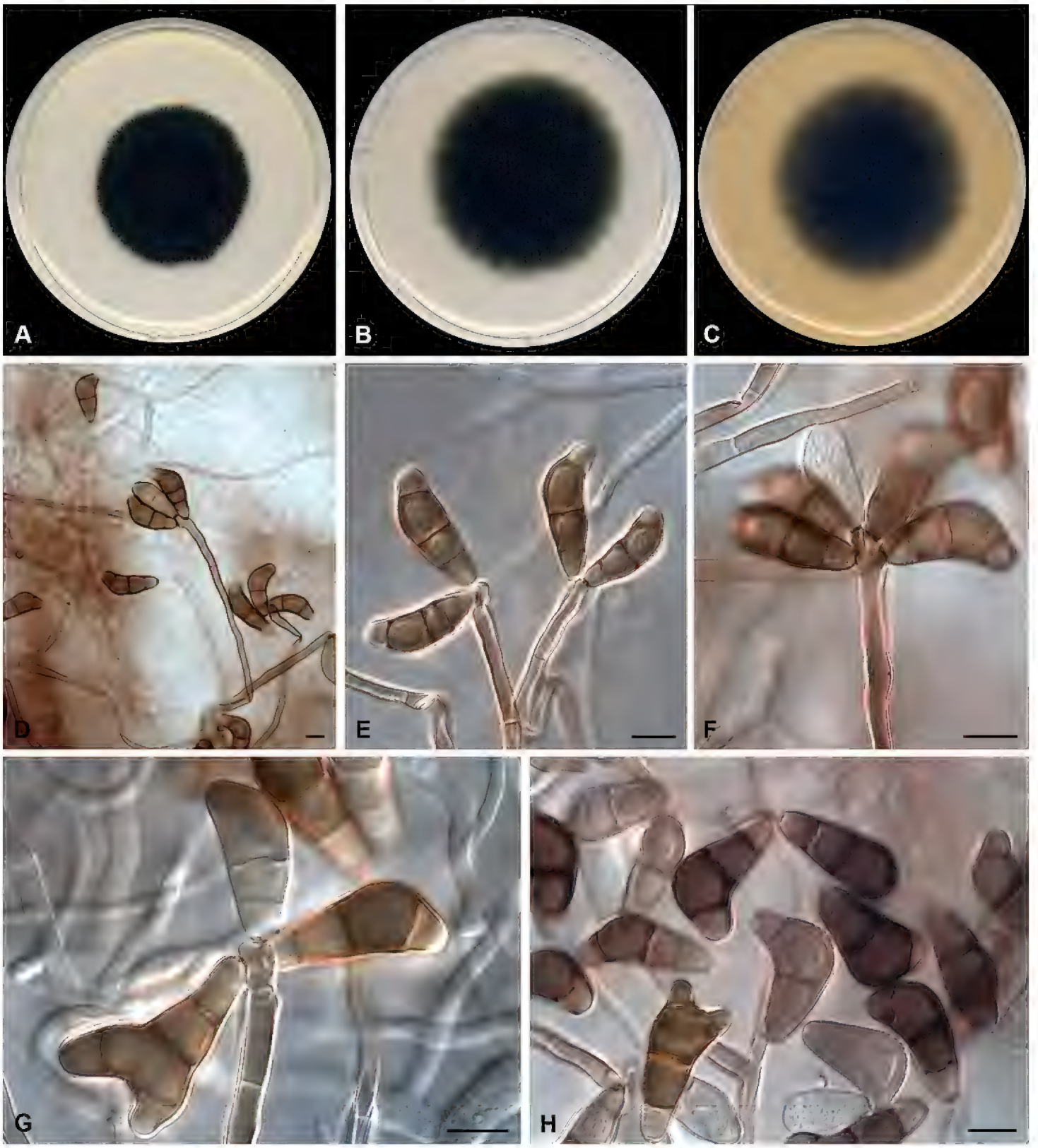

Figure 2. *Curvularia paraverruculosa* sp. nov. (ex-type FMR 17656). **A–C** Colonies on PDA, PCA and OA, respectively, at 25 °C after 7 d **D–H** conidiophores and conidia. Scale bars: 10 µm.

ornamentation. Although conidia in *C. verruculosa*, the closest phylogenetic species and *C. paraverruculosa* are entirely verruculose, they are larger in the former (20–40 × 12–17 µm) (Sivanesan 1987). Furthermore, *C. paraverruculosa* also produces apically bifurcate conidia (Fig. 2), which have not been described in *C. verruculosa*. The conidia of *C. americana* are smaller (13–28 × 7–15 µm) and smooth-walled with a slightly verruculose basal cell (Madrid et al. 2014). In addition, microconidiation, described in *C. americana*, has not been observed in *C. paraverruculosa*.

Curvularia suttoniae **Iturrieta-González, Wiederhold, Gené & Dania García, sp. nov.**
MycoBank No: 833025
Fig. 3

Etymology. Named in honour of the American mycologist Deanna A. Sutton for her contribution to the body knowledge of microfungi.

Type. USA, Texas, from a human leg wound, 2009, *D.A. Sutton* (holotype CBS H-24294, culture ex-type UTHSC 09-3575, CBS 146221, FMR 10992).

Description (PDA at 25 °C). *Mycelium* consisting of branched, septate, pale brown, smooth-walled to verruculose hyphae, 1–4 µm wide. *Conidiophores* mononematous, semi- to macronematous, erect to slightly flexuous, geniculate at the apex, unbranched or branched, smooth-walled to verruculose, pale brown, 43–103 × 3–5 µm (av. (±SD) 80 (±32.35) × 3.7 (±0.67)). *Conidiogenous cells* terminal, subterminal or intercalary, polytretic, proliferating sympodially, pale brown, darkened scars, subcylindrical to slightly swollen, 3–5 µm wide. *Conidia* (2–)3-septate, straight or curved, with the third cell often larger than the rest, apical and middle cells smooth-walled, basal cell verruculose, pale brown to brown, apical and basal cells paler than the middle cells, 8–22 × 5–9 µm (av. (±SD) 15 (±9.89) × 6.88 (±1.18)); hila protuberant, thickened and darkened. Sexual morph not observed.

Culture characteristics (7 d at 25 °C). Colonies on PDA reaching 66–68 mm diam., yellowish-grey (4B2), velvety, flat, margin slightly irregular and fimbriate; reverse black to brownish-orange (5C4); soluble pigment brown (6E6) present in cultures between 30–37 °C. On PCA, reaching 67 mm diam., olive grey (3D2), slightly floccose at the centre, flat, margin regular and whitish; reverse olive grey (3D2), whitish towards periphery. On OA, reaching 64 mm diam., olive grey (3F2), slightly floccose at the centre, flat, margin regular and whitish; reverse olive grey (3F2). Scarce sporulation on the three media.

Cardinal temperature for growth. Optimum 25–30 °C, maximum 37 °C, minimum 5 °C.

Distribution. USA.

Additional specimen examined. USA, South Carolina, from human sphenoid sinus, 2008, *D.A. Sutton* (UTHSC 08-809, FMR 11690).

Notes. *Curvularia suttoniae* is included in a well-supported clade with *C. petersonii* and *C. vietnamensis*, the latter also described here. Although the three species are clearly differentiated phylogenetically (Fig. 1), they can be distinguished only by subtle morphological features. While the conidia of *C. petersonii* and *C. vietnamensis* are entirely smooth, those of *C. suttoniae* show verruculose basal cells. Furthermore, the conidia in *C. petersonii* are narrower (5–7 µm wide) (Tan et al. 2018) and, in *C. vietnamensis*, they are larger (15–28 × 5–12 µm) than those of *C. suttoniae* (8–22 × 5–9 µm). In addition to these morphological features, *gapdh* sequences easily distinguish the two latter species.

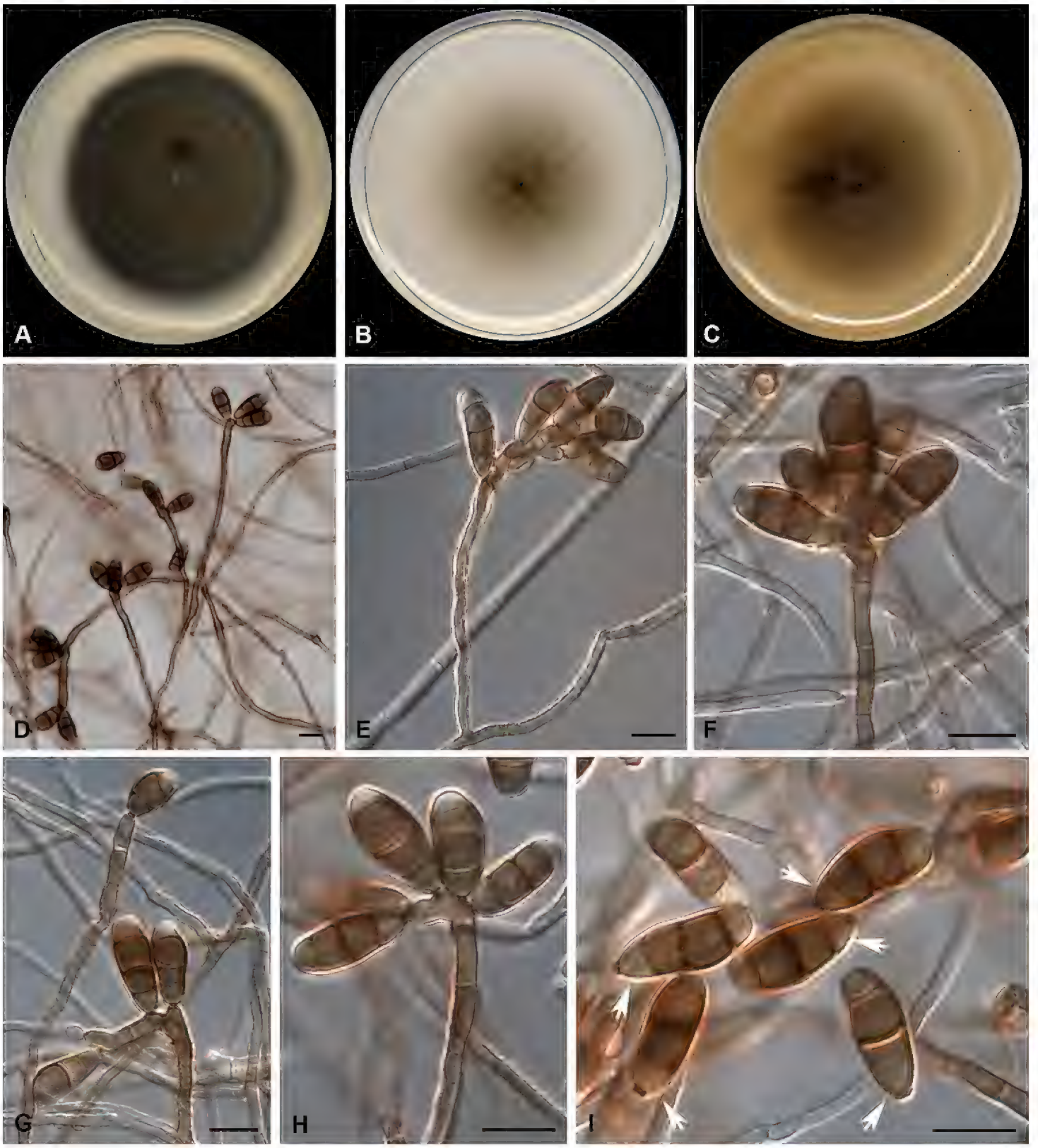

Figure 3. *Curvularia suttoniae* sp. nov. (ex-type FMR 10992). **A–C** Colonies on PDA, PCA and OA, respectively, at 25 °C after 7 d **D–I** conidiophores and conidia with verruculose basal cells (arrows). Scale bars: 10 µm.

Curvularia vietnamensis **Iturrieta-González, Gené & Dania García, sp. nov.**
MycoBank No: 833027
Fig. 4

Etymology. Name refers to the country where the species was collected.

Type. Vietnam, north-east region, on an unidentified dead leaf, Aug 2011, *J. Guarro* (holotype CBS H-24295, culture ex-type CBS 146222, FMR 17659).

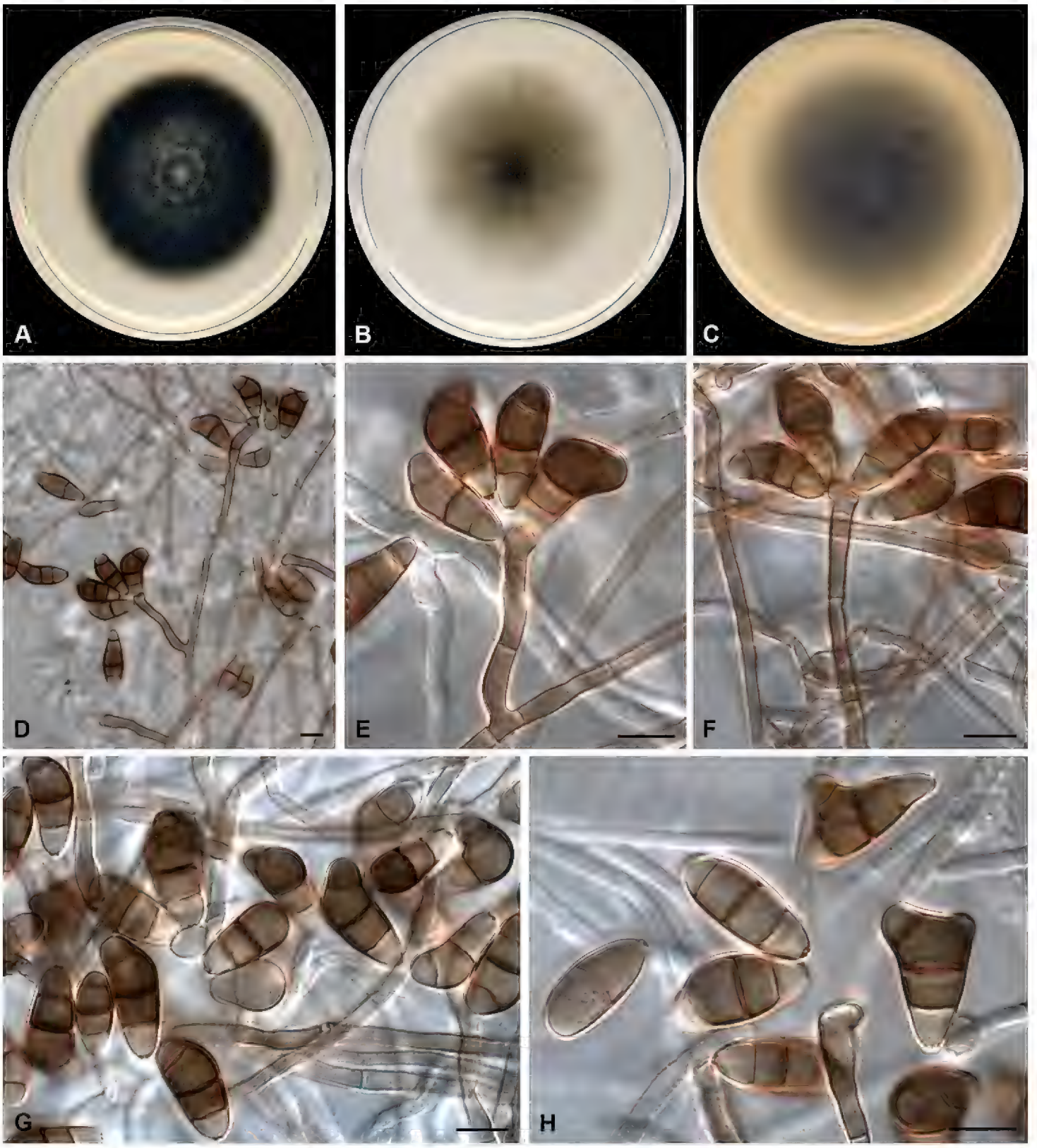

Figure 4. *Curvularia vietnamensis* sp. nov. (ex-type FMR 17659). **A–C** Colonies on PDA, PCA and OA, respectively, at 25 °C after 7 d **D–H** conidiophores and conidia. Scale bars: 10 μm.

Description (PDA at 25 °C). *Mycelium* composed of branched, septate, subhyaline to pale brown, thin and smooth-walled to verruculose hyphae, 2–4 μm wide. *Conidiophores* macronematous, mononematous, septate, straight or flexuous, sometimes slightly geniculate at upper part, unbranched to slightly branched, smooth to verruculose, pale brown to brown, 11–136(–194) × 3–6 μm (av. (±SD) 92.2 (±72.86) × 4.21 (±0.85)). *Conidiogenous cells* terminal or intercalary, mono- or polytretic, proliferating sympodially, pale brown, with darkened scars, subcylindrical to swollen, 3–7 μm wide.

Conidia (1–)3(–4)-septate, curved, with the third cell from base unequally enlarged, some apically bifurcate, smooth-walled, apical and basal cells pale brown, middle cells brown, 15–28 × 5–12 µm (av. (±SD) 21.38 (± 3.44) × 9.34 (±1.83)); hila slightly protuberant, thickened and darkened. Sexual morph not observed.

Culture characteristics (7 d at 25 °C). *Colonies* on PDA reaching 62 mm diam., greenish-grey to dark green (28C2/29F8), final edge white, umbonate, densely floccose, margin regular; reverse grey (29F1), final edge pale grey (1B2). On PCA, reaching 58 mm diam., olive grey to grey (3F2/3B1), slightly floccose at the centre, margin regular, final edge whitish; reverse olive grey to grey (3F2/3B1). On OA, reaching 74 mm diam., olive (2F3) slightly floccose at the centre, margin regular, flat; reverse olive to greenish-grey (2F3/1C2). Sporulation abundant mainly on PCA and OA.

Cardinal temperature for growth. Optimum 30 °C, maximum 37 °C, minimum 15 °C.

Distribution. Indonesia and Vietnam.

Additional specimen examined. Indonesia, from Sorghum seed, 1948, *J. van der Vecht* (CBS 188.48 = FMR 11956).

Notes. See *C. suttoniae* described above.

Discussion

As in other Pleosporalean genera, *Curvularia* is currently a well-delineated genus on the basis of molecular data (Manamgoda et al. 2015, Marin-Felix et al. 2017a). However, morphological features and analyses of the ITS barcode are insufficient to accurately identify *Curvularia* species. Thus, the multi-locus sequence analysis of different gene markers (i.e. LSU, ITS, *gapdh*, *rpb*2 and *tef*1) has been used to study the species diversity in *Curvularia* and phylogentic relationships with other similar genera (Hernández-Restrepo et al. 2018, Manamgoda et al. 2012, 2015, Madrid et al. 2014, Marin-Felix et al. 2017a, 2017b, Tan et al. 2018). Marin-Felix et al. (2017a) regarded ITS, *gapdh* and *tef*1 as the DNA barcodes for species delineation in the genus. During the last three years, numerous new *Curvularia* species have been introduced (Hyde et al. 2017, Marin-Felix et al. 2017a, 2017b, Dehdari et al. 2018, Heidari et al. 2018, Liang et al. 2018, Mehrabi-Koushki et al. 2018, Tan et al. 2018, Tibpromma et al. 2018, Kiss et al. 2019, Raza et al. 2019, Zhang et al. 2020). Novel species are found, not only on fresh material collected in various geographical regions, but also in re-evaluation of *Curvularia* isolates deposited in fungal collections and earlier identified by morphological features or ITS sequence analysis.

The five isolates, studied here, showed morphological similarity with *C. americana* or *C. lunata* (Sivanesan 1987, Madrid et al. 2014), but they also showed subtle variations that did not match with these species. Multi-locus analysis of the recommended barcodes facilitated the delineation of the novel species *C. paraverruculosa*, *C. suttoniae* and *C. vietnamensis*, which were closely related to the known species *C. americana*, *C. petersonii* and *C. verruculosa* (Fig 1).

As in the case of *C. suttoniae*, other related species, such as *C. americana* and *C. verruculosa*, have also been associated with clinical specimens previously (da Cunha et al. 2013, Madrid et al. 2014). However, the role of all these fungi in human diseases has never been proven. Contrary to that, the recently described species *C. coimbatorensis* and *C. tamilnaduensis* were shown to be causal agents of fungal keratitis in India (Kiss et al. 2019). These two latter species, as with *C. suttoniae* and *C. vietnamensis* in our case, could only be molecularly differentiated by *gapdh* and *tef*1 loci; ITS sequence similarity between *C. coimbatorensis* and *C. tamilnaduensis* was 99% (Kiss et al. 2019) and between *C. suttoniae* and *C. vietnamensis*, it was 100%. Therefore, considering clinical laboratories commonly use ITS barcode for fungal diagnosis, not only will the diversity of *Curvularia* species remain obscure in the clinical setting, but also, subsequently, the epidemiology of its species associated with human or animal diseases. Our results suggest that *gapdh* and *tef*1 loci could be good alternatives as barcodes for *Curvularia* identification, since both have a high discriminatory power amongst species. However, *gapdh* would be the recommended locus because there are more sequences available for different species in the genus.

The ITS analysis revealed that *C. palmicola*, only known for its type specimen found on dead branches of *Acoelorrhaphe wrightii* in Thailand (Hyde et al. 2017), is also closely related to the novel species described here. However, this fungus was not included in our concatenate analysis since sequences of *gapdh* and *tef*1 were not available for comparison. Nevertheless, *C. palmicola* can be distinguished morphologically from our species mainly by having conidia with constricted wall at the septum level. Furthermore, *C. palmicola* has longer conidia (23.9–34.7 µm) than *C. suttoniae* (8–22 µm) and C. *vietnamensis* (15–28 µm) and it differs from *C. paraverruculosa* by its smooth-walled conidia.

Despite the fact that DNA sequence analysis is currently mandatory for *Curvularia* identification, two species were recently characterised exclusively, based on morphological data and host association, i.e. *C. tremae* on living leaves of *Trema orientalis* (Haldar 2017) and *C martyniicola* on *Martynia annua* (Kumar and Singh 2018), both from India. *Curvularia tremae* produces up to 4-septate and larger conidia (average length 152.21 µm and 67.75 µm wide at the broadest part) than those described here. Despite the conidia being mostly 3-sepetate, as in our species, *C. martyniicola* differs by having longer conidiophores (95–200 µm) than those of *C. paraverruculosa* (19–85(–145) µm) and *C. suttoniae* (43–103 µm) and by larger conidia (25–45 × 10–15 µm) than those observed in *C. vietnamensis* (15–28 × 5–12 µm).

Acknowledgements

The authors are grateful to Sándor Kocsubé of the University of Szeged (Hungary) for sending the sequences of *C. tamilnaduensis* and *C. coimbatorensis* used in this study. The study was supported by the Spanish Ministerio de Economía y Competitividad, grant CGL2017-88094-P.

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Supplementary material 1

Figure S1. Phylogenetic tree of the genus *Curvularia* based on Maximum Likelihood analysis obtained by RAxML, using the combined analysis of ITS, *gapdh* and *tef*1 and rooted with *Bipolaris maydis* CBS 136.29 and *Bipolaris saccharicola* CBS 155.26

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Data type: phylogenetic tree

Explanation note: Bootstrap values (bs) greater than 70% and Bayesian posterior probabilities (pp) greater than 0.95 are given at the nodes (bs/pp). Bold branches indicate bs/pp of 100/1. The novel species are highlighted in bold. Ex-type and ex-epitype strain are marked with a superscript T and ET, respectively.

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Link: https://doi.org/10.3897/mycokeys.68.51667.suppl1